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GROWTH HORMONE SECRETAGOGUE RECEPTOR IN DOPAMINE NEURONS CONTROLS APPETITIVE AND CONSUMMATORY BEHAVIORS TOWARDS HIGH-FAT DIET IN *AD-LIBITUM* FED MICE

Short title: GHSR in dopamine neurons controls specific appetitive and consummatory behaviors.

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Highlights

- Ghrelin receptor (GHSR) in DA neurons is sufficient to control appetitive and consummatory behaviors towards high fat (HF) diet, in the absence of caloric needs.
- GHSR expression exclusively in DA neurons is sufficient to orchestrate binge-like HF intake.
- GHSR expression exclusively in DA neurons is sufficient to mediate increased anticipatory activity to a scheduled HF diet exposure.

- GHSR expression exclusively in DA neurons is not sufficient to mediate the stimulatory effects of ghrelin treatment on food intake, food seeking and locomotor activity.

Abstract

Growth hormone secretagogue receptor (GHSR), the receptor for ghrelin, is expressed in key brain nuclei that regulate food intake. The dopamine (DA) pathways have long been recognized to play key roles mediating GHSR effects on feeding behaviors. Here, we aimed to determine the role of GHSR in DA neurons controlling appetitive and consummatory behaviors towards high fat (HF) diet. For this purpose, we crossed reactivable GHSR-deficient mice with DA transporter (DAT)-Cre mice, which express Cre recombinase under the DAT promoter that is active exclusively in DA neurons, to generate mice with GHSR expression limited to DA neurons (DAT-GHSR mice). We found that DAT-GHSR mice show an increase of c-Fos levels in brain areas containing DA neurons after ghrelin treatment, in a similar fashion as seen in wild-type mice; however, they did not increase food intake or locomotor activity in response to systemically- or centrally-administered ghrelin. In addition, we found that satiated DAT-GHSR mice displayed both anticipatory activity to scheduled HF diet exposure and HF intake in a binge-like eating protocol similar to those in wild-type mice, whereas GHSR-deficient mice displayed impaired responses. We conclude that GHSR expression in DA neurons is sufficient to both mediate increased anticipatory activity to a scheduled HF diet exposure and fully orchestrate binge-like HF intake, but it is insufficient to restore the acute orexigenic or locomotor effects of ghrelin treatment. Thus, GHSR in DA neurons affects appetitive and consummatory behaviors towards HF diet that take place in the absence of caloric needs.

Keywords: GHSR, dopamine, high-fat, mesocorticolimbic pathway, ghrelin, appetite.

Introduction

The stomach-derived hormone ghrelin controls a variety of feeding behaviors (Perello and Dickson, 2015; Yanagi et al., 2018). Ghrelin acts via the growth hormone secretagogue receptor (GHSR), a G protein-coupled receptor highly expressed in most brain areas involved in food intake regulation (Zigman et al., 2006). Ghrelin's actions on food intake include rapid orexigenic effects, which are observed after ghrelin treatment in both satiated human beings and *ad libitum* fed mice exposed to regular chow (McFarlane et al., 2014; Wren et al., 2001). In mice, acute orexigenic

effects of ghrelin are mainly mediated by a subset of neurons of the hypothalamic arcuate nucleus (ARC) that synthesize the orexigenic neuropeptides agouti-gene-related protein and neuropeptide Y ($ARC^{AgRP/NPY}$ neurons) and express high levels of GHSR (Nakazato et al., 2001; Willesen et al., 1999). Also, ghrelin affects other more sophisticated feeding-related appetitive behaviors including food seeking, food preference, food reward, reinforcement or motivation. Indeed, functional magnetic resonance imaging studies in human beings have shown that ghrelin modulates brain activity in areas controlling appetitive behaviors (Malik et al., 2008). Ghrelin's orchestration of these complex behaviors would favor animals to seek and consume some types of palatable foods (Perello and Dickson, 2015). In this regard, a variety of studies has shown that ghrelin signaling affects appetitive and consummatory behaviors towards high fat (HF) diet in rodents. Ghrelin administration shifts food preference towards a HF diet or lard consumption in rats (Shimbara et al., 2004). In mice, ghrelin treatment also increases the rewarding value of HF diet and the motivation to obtain it (Perello et al., 2010). GHSR-deficient mice eat similar amounts of HF diet than wild-type controls in *ad libitum* fed conditions (Perello et al., 2010; Sun et al., 2008; Zigman et al., 2005); however, GHSR-deficient mice display a smaller tendency to eat HF diet under particular conditions. For instance, GHSR-deficient mice lack conditioned place preference to HF diet under calorie restriction, contrary to what is observed in wild-type mice (Perello et al., 2010). Mice lacking ghrelin show less motivation to work for a HF diet and a decreased HF diet intake in a 'dessert' protocol, in which the palatable diet is provided to calorically-satiated mice after a fasting event (Davis et al., 2012). *Ad libitum* fed GHSR-deficient mice also eat less HF diet, as compared to control littermates, when the palatable diet is time-limited provided either in a daily or intermittent fashion (Valdivia et al., 2015; King et al., 2016). The specific neuronal populations by which GHSR regulates such non-homeostatic aspects of HF intake have not been identified.

The dopamine (DA) pathways play key roles mediating GHSR effects on feeding behaviors. The major DA pathways include the mesocorticolimbic, the nigrostriatal and the tuberoinfundibular pathways. The mesocorticolimbic pathway refers to the DA neurons of the ventral tegmental area (VTA^{DA}) that project to the nucleus accumbens (Acb) as well as to other cortical brain areas (e.g. prefrontal cortex) and regulate a variety of functions related to reward such as incentive salience, motivation and reinforcement learning, among other cognitive processes (Koob and Volkow, 2016). The nigrostriatal pathway refers to the DA neurons of the substantia nigra pars compacta (SN^{DA}) that project to the dorsal striatum and mainly regulate voluntary movement through basal ganglia motor loops (Sonne and Beato, 2020). The tuberoinfundibular pathway refers to the DA neurons of the arcuate nucleus (ARC^{DA}) that project to the median eminence and control prolactin release from the pituitary. The combined immuno-labeling of tyrosine hydroxylase (TH), which is the gold

standard method to identify catecholamine-producing neurons, with either *in situ* hybridization histochemistry against GHSR mRNA or labeling strategies using fluorescent ghrelin have shown that VTA^{DA}, SN^{DA} and ARC^{DA} neurons express GHSR (Abizaid et al., 2006; Zigman et al., 2006). Electrophysiological recordings have shown that DA neurons of these brain areas are directly activated by ghrelin (Abizaid et al., 2006; Zhang and van den Pol, 2016). As an attempt to clarify the food intake-related implications of GHSR in DA pathways, some studies performed intra-nuclei administration of ghrelin. For instance, intra-VTA ghrelin treatment increases Acb DA release, regular chow intake and locomotor activity (Cornejo et al., 2018a; Jerlhag et al., 2006; Kawahara et al., 2009). Intra-VTA administration of ghrelin to food-restricted rats enhances, while chronic intra-VTA administration of a GHSR antagonist blunts, operant responding for chocolate pellets (King et al., 2011). Also, chronic intra-VTA administration of ghrelin increases cue-induced reinstatement of lever pressings to obtain chocolate pellets in food-restricted rats (St-Onge et al., 2016). Intra-VTA administration of a GHSR antagonist selectively reduces HF diet intake while does not affect the intake of less-preferred high protein or high carbohydrate diets (King et al., 2011). Furthermore, VTA-lesioned rats spend less time than control rats exploring tubes containing peanut butter in response to centrally-administered ghrelin (Egecioglu et al., 2010). However, these manipulations are strongly conditioned by the precision of the injections and lack of cellular specificity since injected ghrelin impacts not only DA neurons but also other non-DA neurons of the VTA that also express GHSR, such as GABA neurons (Cornejo et al., 2018a).

In order to clarify the role of GHSR in DA neurons, we took advance of the cre/loxP site-specific recombinase system to generate mice with GHSR expression limited to DA neurons. Specifically, we used GHSR-deficient mice that lack GHSR because they harbor a loxP-flanked transcriptional blocker inserted into the GHSR gene but can re-express GHSR, in a given cell population, following Cre-mediated DNA recombination (Zigman et al., 2005). GHSR-deficient mice were crossed with DA transporter (DAT)-Cre mice, which express Cre under the DAT promoter that is active exclusively in DA neurons (Lorang et al., 1994). Thus, the subset of mice derived from these crosses that harbor two alleles of the mutant GHSR gene and express Cre under the DAT promoter (hereafter named DAT-GHSR mice) should express GHSR exclusively in DAT-expressing neurons. Here, we studied DAT-GHSR mice under different experimental paradigms to assess if GHSR expression in DA neurons is sufficient to control appetitive and consummatory behaviors towards HF diet in the absence of caloric needs.

Materials and methods

Animals. This study was performed with adult (9-12 weeks old) male mice generated at the animal care facility of the IMBICE and housed under a 12-h light/dark cycle with water and chow available *ad libitum*, except when indicated (Gepsa, Grupo Pilar, www.gepsa.com; chow provided 3.0 kcal/g energy and its percent weight composition was: proteins 25.5 g%, fat 3.6 g%, carbohydrates 28.8 g%, fibers 27.4 g%, minerals 8.1 g% and water content 6.7 g%). The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA. The protocol was approved by the Institutional Animal Care and Use Committee of the IMBICE (approval ID 10-0122). Experimental mice included: 1) Wild-type (WT) C57BL6 mice, 2) DAT-tdTomato mice, 3) GHSR-deficient mice and 4) DAT-GHSR mice. DAT-tdTomato mice were generated by crossing DAT-Cre mice with Ai14 mice. DAT-Cre mice (Jackson Laboratory, B6.SJL-*Slc6a3*$^{tm1.1(cre)/Bkmn}$/J, Stock #: 006660) express an internal ribosome entry site-linked Cre recombinase downstream the endogenous *Slc6a3* gene (Bäckman et al., 2006). Ai14 mice (Allen Institute, 129S6-Gt(ROSA)26Sor$^{tm14(CAGtdTomato)Hze}$/J, Stock #: 007908) harbor a targeted mutation of the Gt(ROSA)26Sor locus with a loxP-flanked STOP cassette preventing the transcription of tdTomato, which is expressed only following Cre-mediated recombination (Madisen et al., 2010). GHSR-deficient mice contain a loxP-flanked transcriptional blocker inserted into the intron located downstream of the transcriptional start site and upstream of the translational start site of the *ghsr* gene (Zigman et al., 2005). The transcriptional blocking cassette inserted in the GHSR-deficient mice includes: i) a splice acceptor site from the mouse engrailed 2 gene followed by an SV40 poly(A) signal, ii) an SV40 enhancer followed by a neomycin resistance gene and two HSV-TK poly(A) signals, iii) a synthetic poly(A) signal/transcriptional pause signal, and iv) another synthetic poly(A) signal followed by a Myc-associated zinc finger protein-binding site (Zigman et al., 2005). This nonstandard, knockout approach enables Cre recombinase–mediated reactivation of GHSR expression in a specific cell type. DAT-GHSR mice were generated by crossing GHSR-deficient mice with DAT-Cre mice.

Drugs. Ghrelin (Global Peptides, cat. PI-G-03) was dissolved in phosphate buffered saline (PBS, pH 7.4) and prepared fresh on each experimental day. For subcutaneous (SC) administrations, ghrelin was injected at a dose of 60 pmol/g body weight (BW), which elevates plasma ghrelin levels to concentrations reached under energy deficit conditions and robustly increases food intake (Cabral et al., 2016; Chuang et al., 2011). For central administrations (intra-cerebro-ventricular, ICV), ghrelin was injected at a dose of 60 pmol/mouse, the minimum dose that induce a robust increase in food intake in our experimental conditions.

Quantification of GHSR mRNA levels in brain nuclei. As we have done in the past (Fernandez et al., 2018), we quantified the levels of GHSR mRNA by qRT-PCR. Briefly, brains from WT (n=4), GHSR-deficient (n=4) and DAT-GHSR (n=4) mice were extracted after decapitation, sectioned into 1 mm coronal slices and punches corresponding to the ARC, VTA/SN and dentate gyrus (DG) were excised with a 1 mm micropuncher. Punches were collected in TRIzol Reagent (Thermo Scientific, cat. 15596018), total RNA was isolated and reverse-transcribed into cDNA using random hexamer primers and MMLV reverse transcriptase (Thermo Scientific, cat. 28025021). Quantitative PCR for GHSR in each region was performed in duplicate with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, cat. 08-24-00001) using a StepOne Cycler. Fold change from WT values was determined using the $2^{-\Delta\Delta Ct}$ method. Primers sequences for GHSR were sense: 5′-GCTCTGCAAACTCTTCCA-3′, antisense: 5′-AAGCAGATGGCGAAGTAG-3′ [GenBank Accession No. NM_177330.4], product size 99 bp. Primers sequences for ribosomal protein L19 (housekeeping) were sense: 5′-AGCCTGTGACTGTCCATTCC-3′, antisense: 5′-TGGCAGTACCCTTCCTCTTC-3′ [GenBank Accession No. NM_009078.2], product size 99 bp.

Experimental design for behavioral assessments. Six cohorts of mice, which included a total of 38 WT, 27 GHSR-deficient and 31 DAT-GHSR male mice, were used for behavioral assessments. Initially, mice were individually housed 3 days before any experiment. Four independent cohorts were successively used as follows: 1) food intake assessment in response to systemic treatment, 2) exposure to HF diet binge eating protocol, 3) implantation of ICV cannulas, 4) food seeking or locomotor activity assessment in response to ICV treatment, and 5) food intake assessment in response to ICV treatment and perfusion. Additionally, two independent cohorts were used to assess HF diet anticipatory activity. Mice were allowed to recover after each treatment as indicated below. Of note, the exact number of mice for each assessment is also indicated below and slightly differed among assessments due the different reasons (e.g. limited availability of monitoring boxes, 3 mice were euthanized because displayed signs of illness after ICV surgery; 7 brains were not adequately fixed and were not used for immunostaining).

Food intake assessment in response to systemic treatment. Food intake was assessed in *ad libitum* fed WT (n=9 vehicle and n=16 ghrelin), GHSR-deficient (n=3 vehicle and n=3 ghrelin) and DAT-GHSR (n=10 vehicle and N=13 ghrelin) mice. On the experimental day, food was removed from the home cage hoppers and mice were SC administered with either vehicle (saline) or ghrelin. After SC administrations, mice were exposed to a single pre-weighed standard diet pellet and remained undisturbed for 2 h, when pellets and any additional spillage were collected

and weighed. Two-hour food intake was calculated subtracting the remaining weight of the standard diet pellet to the initial weight, and expressed in mg.

HF diet binge eating protocol. Individually-housed WT (n=23), GHSR-deficient (n=18) and DAT-GHSR (n=16) mice were allowed to recover for one week after the SC administrations and used as we have done before (Cornejo et al., 2019; Valdivia et al., 2015). At 9.00 am of experimental day 1 mice were exposed to a pellet of HF diet (Gepsa, Grupo Pilar, www.gepsa.com) which provided 3.9 kcal/g energy and its percentage weight composition was: proteins 22.8 g%, fat 21.1 g%, carbohydrates 22.5 g%, fiber 23.0 g%, minerals 5.6 g% and water content 5.0 g%. Mice remained undisturbed until 11.00 am, when the HF pellet was removed and weighed. This procedure was repeated for 4 consecutive days and daily HF diet intake in the 2-h HF diet access was calculated by subtracting the weight of the remaining HF diet pellet at 11.00 am to the weight of the initial HF diet pellet.

ICV cannula implantation. Three days after the binge eating protocol, mice were deeply anesthetized with ketamine/xylazine and stereotaxically implanted with an ICV guide cannula (PlasticsOne) in the lateral ventricle (placement coordinates: anteroposterior: −0.34 mm, mediolateral: +1.0 mm and dorsoventral: −2.3 mm). Then, mice were allowed to recover prior to the experimental day and, before any experimental procedure involving ICV administrations, mice were acclimated to handling by removing the dummy cannula and connecting an empty cannula connector. Injections were made using a 30-gauge injector cannula and the correct location of the cannulas was verified post-mortem in brain sections.

Food seeking assessment. Food seeking was assessed in WT (n=8), GHSR-deficient (n=4) and DAT-GHSR (n=4) mice previously implanted with an ICV guide cannula and allowed to recover for, at least, 3 days after surgery. Mice were placed in recording cages that, in turn, were allocated in a ventilated and acoustically isolated monitoring box equipped with an overhead camera and dimmable LED illumination. Mice were individually housed before the experiment in the recording cages and had standard diet available *ad libitum*, except when transferred to the monitoring box. Before assessing locomotor activity, mice were habituated to the recording environment by placing them in the monitoring box during 30 min on 2 consecutive days. On the experimental day, mice were placed in the monitoring box for 40 min after they were ICV administered with either vehicle (artificial cerebrospinal fluid, aCSF) or ghrelin on consecutive days in a crossover design fashion. Mice activity was recorded and videos were imported into Fiji (Cornejo et al., 2018a; Schindelin et al., 2012), sampled at 8 frames per second and processed to extract food seeking behavior,

defined as digging in the bedding with front legs and nose for more than 5 seconds, which was expressed in minutes.

Locomotor activity assessment. Locomotor activity was assessed in WT (n=15), GHSR-deficient (n=6) and DAT-GHSR (n=7) mice previously implanted with an ICV guide cannula as we have done in the past (Cornejo et al., 2018a). Briefly, mice were placed in recording cages and, in turn, transferred to the monitoring box. Before assessing locomotor activity, mice were habituated to the recording environment by placing them in the monitoring box during 1 h on 2 consecutive days. On the experimental day, mice were transferred to the recording cage and placed in the monitoring box for 40 min after they were ICV administered with either aCSF or ghrelin on consecutive days in a crossover design fashion. Locomotor activity was registered for 35 min after treatment, videos were processed as described before and total distance traveled was extracted and expressed in meters.

Food intake assessment in response to ICV treatment. Food intake was assessed in *ad libitum* fed WT (n=7 vehicle and n=17 ghrelin), GHSR-deficient (n=8 vehicle and n=9 ghrelin) and DAT-GHSR (n=5 vehicle and N=16 ghrelin) mice allowed to recover for 1 week after previous ICV treatment. On the experimental day, food was removed from the home cage hoppers and mice were ICV administered with either aCSF or ghrelin. After ICV administrations, the procedure was similar as described for SC administrations. Two hours after treatment, mice were anesthetized and transcardially perfused, their brains were obtained and processed for immunostainings as detailed below.

HF diet anticipatory activity. WT (n=13), GHSR-deficient (n=9) and DAT-GHSR (n=8) mice were first habituated to a 2-chamber polycarbonate apparatus in which the experiments were performed. Each chamber (25 x 30 x 15.5 cm) of the apparatus has visual and tactile cues that allow animals to differentiate between chambers and a gate that connects both chambers. The 2-chamber apparatus was adapted to home cages and the same monitoring box used for locomotor activity and food seeking experiments was used to register mice behavior. Initially, mice were placed and monitored in the 2-chamber apparatus in order to corroborate there was no bias in the time spent in each chamber (pre-test). Then, mice were subjected to 1 h conditioning sessions on 3 consecutive days to associate one chamber with HF diet (Research Diets, cat. D12331) which provided 5.56 kcal/g energy and its percentage weight composition was: protein 23.0 g%, fat 35.8 g%, carbohydrates 35.4 g%, minerals 5.4 g% and vitamins 0.3 g%. On test day, mice were placed for 45 min in the 2-chamber apparatus without access to food and their behavior was recorded.

Videos were imported into Fiji (Cornejo et al., 2018a), sampled at 8 frames per second and processed using custom-made macros to extract total distance traveled and time spent in each chamber, which were expressed in meters and seconds, respectively.

Immunostainings. For the immunostainings, we proceed as in the past (Cornejo et al., 2018a). Briefly, brains of transcardially perfused mice were obtained, frozen and coronally cut at 40 µm. For the fluorescent anti-tyrosine hydroxylase (TH) immunostaining, brain sections of DAT-tdTomato mice (n=2) were treated with blocking solution (3 % normal donkey serum in 0.25 % Triton-X in PBS) and then incubated with an anti-TH antibody (Santa Cruz, cat. SC-14007, 1:5,000) for 48 h at 4°C. Finally, sections were incubated with an Alexa Fluor594-conjugated anti-rabbit antibody (Invitrogen, cat. A11008, 1:1000). Brain sections were mounted on glass slides and coverslipped with mounting media. For double chromogenic anti-c-Fos and anti-TH immunostaining, WT (vehicle n=7 and ghrelin n=17), GHSR-deficient (vehicle n=8 and ghrelin n=9) and DAT-GHSR (vehicle n=4 and ghrelin n=10) mice were perfused 2 h after ICV administrations and their brains were obtained and cut. Here, sections were treated with 0.5% H_2O_2, treated with blocking solution and then incubated with a rabbit anti-c-Fos antibody (Santa Cruz, cat. SC-7202; 1:2,000) for 48 h. Next, sections were sequentially incubated with a biotinylated donkey anti-rabbit antibody (Vector Laboratories, cat# BA-1000, 1:1,000), reagents of the Vectastain Elite ABC kit and a DAB/nickel solution that generates a black nuclear precipitate in c-Fos+ cells. Then, sections were incubated with the anti-TH antibody (1:20,000) for 48 h and sequentially incubated with the biotinylated donkey anti-rabbit antibody, reagents of the Vectastain Elite ABC kit, and a DAB solution without nickel, which generates a brown cytoplasmic precipitate in TH+ cells. Sections were mounted on glass slides and coverslipped with mounting media.

Quantitative neuroanatomical analysis. Blind quantitative analysis was performed independently by two observers under the same optical conditions. Quantitative analysis was performed in sections between bregma 0.86 and 1.18 mm for the Acb, between bregma -0.34 and -0.82 mm for the suprachiasmatic nucleus (SCh), between bregma -1.22 and -1.94 mm for the ARC, between bregma -2.30 and -2.70 mm for the premammillary ventral nucleus (PMV), between bregma -3.28 and -3.80 mm for the VTA and between bregma -2.08 and -3.80 for the SN. Anatomical limits of each brain region were identified using a mouse brain atlas (Paxinos and Franklin, 2001). All quantitative neuroanatomical analyses were corrected for double counting according to the method of Abercrombie, as we have done in the past (Cornejo et al., 2018a). The total number of immunoreactive (denoted with a + symbol) was calculated using the formula of

Konigsmark, where the total number of cells is equal to the number of cells counted per section multiplied by the total number of sections through the nucleus (Cornejo et al., 2018a).

Statistical analyses. Data are expressed as the mean±standard error of the mean (SEM). Normality was tested using the D'Agostino & Pearson omnibus test and homogeneity of variances, using Bartlett's test. The statistical tests and post-tests used considered sample size, normality of distribution and homogeneity of variances of each dataset and are reported in the figure captions, as well as the statistical outcomes obtained in each case. Differences were considered significant when $p<0.05$.

Results

First, we performed a systematic anatomical analysis of Cre expression in DAT-Cre mice in our experimental conditions. Cre-expressing neurons were visualized by crossing DAT-Cre mice and reporter mice, which express the red fluorescent tdTomato protein only following Cre-mediated recombination. Mice generated from these crosses, which were named DAT-tdTomato mice, had ~1300, ~11500 and ~8000 tdTomato+ neurons in the ARC, the VTA and the SN, respectively (Fig. 1A). DAT-tdTomato mice lacked tdTomato+ neurons in the rest of the brain, except for the SCh and PMV, where ~500 and ~1900 tdTomato+ neurons were respectively detected. In order to confirm that tdTomato+ neurons were DA neurons, we performed a green fluorescent immunostaining against TH in brain sections of DAT-tdTomato mice (Fig. 1A). Quantitative analysis of the degree of co-localization indicated that double tdTomato+/TH+ cells represented 82±6, 96±2 and 98±1 % of the total TH+ cells of the ARC, VTA and SN, respectively, and represented 94±5, 94±2 and 95±2 % of the total tdTomato+ cells of the ARC, VTA and SN, respectively. No TH+ cells were found in the SCh or the PMV. Thus, we confirmed previous reports showing that DAT-Cre mice are a valuable tool to perform specific genetic manipulations in DA neurons (Lammel et al., 2015; Stamatakis et al., 2013).

After validation of DAT-Cre mice, we generated DAT-GHSR mice, which should express GHSR exclusively in DAT-expressing neurons programmed to express GHSR. WT, GHSR-deficient and DAT-GHSR mice displayed similar daily food intake and body weight gain under standard chow diet (not shown). In order to assess GHSR mRNA expression, we used qRT-PCR on tissue punches from different brain regions of these mice. GHSR mRNA levels are highest in the ARC followed by the VTA (~70 % relative to ARC), GHSR mRNA levels are low in the DG (~10 % relative to the ARC) and undetectable in the PMV and SCh (Chuang et al., 2011). We found that GHSR mRNA levels in the ARC and the VTA/SN of DAT-GHSR mice were higher than in GHSR-deficient mice (Fig. 1B-C). Importantly, DAT-GHSR mice did not significantly restore GHSR mRNA expression in brain areas that lack Cre-expressing cells, such as the DG (20±3 vs 100±10 % for DAT-GHSR mice and WT mice, respectively).

We then investigated the effect of ghrelin treatment on DAT-GHSR mice. First, we tested the effect of systemically administered ghrelin in a dose that mainly impacts on the ARC and increases food intake, but not locomotor activity, in WT mice (Cabral et al., 2015; Cornejo et al., 2018a). As seen in GHSR-deficient mice, DAT-GHSR mice did not increase food intake in response to SC-injected ghrelin (Fig. 2A). Then, we tested the effect of centrally administered

ghrelin using experimental conditions that are known to increase food intake and locomotor activity in WT mice (Cornejo et al., 2018a). As seen in GHSR-deficient mice, DAT-GHSR mice did not increase either food intake, the time spent digging or locomotor activity in response to ICV-injected ghrelin (Fig. 2B-D). In order to assess the capability of ghrelin to act in DA neurons of DAT-GHSR mice, we performed double immunostaining against TH and the marker of neuronal activation c-Fos in brain sections of WT, GHSR-deficient and DAT-GHSR mice that had been ICV-treated with vehicle or ghrelin (Fig. 3A). WT, GHSR-deficient and DAT-GHSR mice had similar number of TH+ cells in the ARC (1290±57, 992±71 and 970±109 cells, respectively), the VTA (7713±609, 8193±516 and 9304±738 cells, respectively) and the SN (6075±310, 6107±387 and 6234±566 cells, respectively). As previously shown, ghrelin-treated WT mice displayed an increase of the number of c-Fos+ cells in the ARC, the VTA, the SN and the Acb, as compared to vehicle-injected WT mice (Fig. 3B-E). Ghrelin-treated WT mice also showed an increase of the fraction of c-Fos+/TH+ cells in the ARC as compared to vehicle-injected WT mice (8.2±2.3 vs 0.0±0.0 %, respectively. Two-way ANOVA: $F(1,49)_{treatment}=4.191$, p=0.0460; Dunnett's T3: p=0.0385). In the VTA and SN, the fraction of c-Fos+/TH+ cells tended to increase but the comparison did not reach statistical significance (2.5±1.0 vs 1.3±0.9 and 0.7±0.3 vs 0.0±0.0 %, respectively, as compared to vehicle-injected WT mice). In contrast, ghrelin-treated GHSR-deficient mice did not change the number of c-Fos+ cells in the ARC, the VTA, the SN and the Acb (Fig. 3B-E) nor the fraction of c-Fos+/TH+ cells in the ARC, the VTA or the SN (0.8±0.4 vs 0.9±0.4, 0.4±0.2 vs 0.3±0.1 and 0.0±0.0 vs 0.0±0.0 %, respectively, as compared to vehicle-injected GHSR-deficient mice). Ghrelin-treated DAT-GHSR mice displayed an increase of the number of c-Fos+ cells in the ARC, the VTA, the SN and the Acb, as compared to vehicle-injected DAT-GHSR mice (Fig. 3B-E), while the fraction of c-Fos+/TH+ cells in the ARC, the VTA and the SN remained unchanged (2.3±0.8 vs 0.1±0.1, 0.5±0.1 vs 0.0±0.0 and 0.5±0.3 vs 0.0±0.0 %, respectively, as compared to vehicle-injected DAT-GHSR mice).

We then studied if the expression of GHSR in DA neurons is sufficient to restore some rewarding behaviors that are known to be impaired in GHSR-deficient mice. In a set of experiments, satiated mice were conditioned to associate one chamber with HF diet (conditioning period) in a 2-chamber apparatus (Fig. 4A). On the test day, mice were permitted free access to both chambers in the absence of food, and the locomotor activity as well as the time spent in each chamber were quantified. As predicted, we found that WT mice conditioned to receive HF diet in one of the chambers traveled more distance, in anticipation to the palatable stimulus, as compared to WT mice conditioned to receive regular chow (4.5±1.8 vs. 2.4±0.4 m, respectively; unpaired t-test, $t(16)=5.301$, $p<0.0001$). In addition, WT mice conditioned to receive HF diet spent more time in the

chamber that they associated with food (58±3 vs. 42±3 % of the total time; paired t-test, t(12)=2.230, p=0.0456), while WT mice conditioned to receive regular chow spent similar time in each chamber (51±5 vs. 49±4 % of the total time). When comparing the anticipatory activity to HF diet exposure among the different groups of mice, we found that WT and DAT-GHSR mice traveled a similar distance, while GHSR-deficient mice showed a 19±6 % reduction of the total distance traveled as compared to both WT mice and DAT-GHSR mice (Fig. 4B). WT mice conditioned to receive a HF diet spent more time in the HF diet-paired chamber, while GHSR-deficient and DAT-GHSR mice spent similar amounts of time in each chamber in the test day (Fig. 4C).

In an independent study, satiated mice were daily exposed to a HF diet pellet during 2-h for four consecutive days (Fig. 4D). WT mice showed an escalating profile of 2-h HF intake over the successive days (Fig. 4E-F), as previously reported (De Francesco et al., 2019; Valdivia et al., 2015). GHSR-deficient mice also ate HF diet every day, but they displayed a 24±6 % smaller cumulative 4-day HF intake, as compared to WT mice, over the four days of the experiment (Fig. 4E-F). Notably, DAT-GHSR mice showed daily 2-h HF intake, escalation profile of 2-h HF intake and cumulative 4-day HF intake similar as WT mice (Fig. 4E-F).

Discussion

The current study helps to clarify the specific role of GHSR in DA neurons. In particular, we found that the presence of GHSR exclusively in DA neurons of the mouse brain is not sufficient to mediate the stimulatory effects of ghrelin treatment on food intake, food seeking or locomotor activity. However, the presence of GHSR in DA neurons is sufficient to restore more elaborated reward-related eating behaviors, which are otherwise impaired in GHSR-deficient mice under the same experimental conditions.

Conditional GHSR-deficient mice have been instrumental in investigating the role of GHSR in specific neuronal types. GHSR-deficient mouse is a non-standard knock-out model, which contains a transcription blocking cassette that can be removed in a Cre-dependent manner. Such transcriptional blocker not only impairs *ghsr* gene expression but also contains a splice acceptor site that ensures that generated “read-through” transcripts result in chimeric mRNA that does not generate a functional GHSR protein. Here, we confirmed our previous observations that GHSR-deficient mice are unresponsive to ghrelin treatment despite they show a markedly reduced, but detectable, GHSR mRNA expression in some brain areas (Chuang et al., 2011). In the past, we had crossed GHSR-deficient mice with TH-Cre mice in order to generate TH-GHSR mice and try to elucidate the role of GHSR in DA and other catecholaminergic neurons (Chuang et al., 2011). TH-GHSR mice displayed GHSR expression not only in the ARC, the VTA and the SN but also in other areas of the hypothalamus (e.g. anteroventral periventricular nucleus, dorsomedial nucleus and ventromedial nucleus) and caudal brainstem (e.g. nucleus of the solitary tract). TH-GHSR mice showed partial restoration of ghrelin-induced food intake and ghrelin-induced HF diet conditioned place preference (Chuang et al., 2011). However, further studies showed that TH-Cre mice induces substantial recombination in non-catecholaminergic neurons because TH is transiently expressed during development in some cells that no longer express TH in adulthood and also because TH promoter can drive ectopic Cre expression (Lammel et al., 2015; Lindeberg et al., 2004). For instance, TH-GHSR mice should not express GHSR in the nucleus of the solitary tract, as TH+ neurons lack GHSR in adult mice (Cornejo et al., 2018b). Thus, TH-GHSR mice may display GHSR expression in unintentionally targeted cells that may confound the observations of the supposedly cell-type-specific experiments (Lammel et al., 2015). Here, we used DAT-Cre mice to manipulate *ghsr* gene expression in DA neurons. First, we used immnunostaining against TH, which is the gold standard and the most widely accepted method for identifying DA neurons (Papathanou et al., 2019; Yip et al., 2018), to confirm previous studies showing that DAT-Cre mice display a selective and specific expression of Cre in DA neurons (Lammel et al., 2015). In addition, we found

that DAT-GHSR mice express GHSR exclusively in the ARC, the VTA and the SN. Thus, DAT-GHSR mice represent a valuable model to more precisely clarify the role of GHSR in DA neurons.

Current results provide new insights about the role of ghrelin-evoked GHSR signaling in the different sets of DA neurons. ARC^{DA} neurons could sense fluctuations of plasma ghrelin levels because they are located nearby the fenestrated capillaries that branch from the hypophyseal system (Perello et al., 2019). Here, we found that DAT-GHSR mice do not increase food intake in response to systemically injected ghrelin suggesting that ARC^{DA} neurons do not mediate the orexigenic effect of plasma ghrelin. ARC^{DA} neurons release DA in the median eminence that, in turns, inhibits the release of prolactin from pituitary lactotroph cells. Here, we confirmed previous studies showing that ghrelin treatment increases c-Fos in ARC^{DA} neurons (Pirnik et al., 2014), although this effect seems to be indirect. Further studies are required to test if ghrelin action exclusively in ARC^{DA} neurons is sufficient to regulate the neuroendocrine effects of this neuronal population. Interestingly, a study showed that some TH+ neurons of the ARC in mice also innervate some hypothalamic nuclei and increase food intake (Zhang and van den Pol, 2016). Our results suggest that these presumably orexigenic ARC^{DA} neurons are not directly activated by ghrelin.

In the midbrain, a subpopulation of VTA^{DA} and SN^{DA} neurons express GHSR (Andrews et al., 2009; Chuang et al., 2011; Zigman et al., 2006). Here, we found that some VTA and SN neurons of the DAT-GHSR mice increase c-Fos expression in response to centrally-injected ghrelin suggesting that ghrelin present in the cerebrospinal fluid can directly activate some midbrain DA neurons. Notably, a small fraction of VTA^{DA} and SN^{DA} neurons increase c-Fos expression in WT mice ICV-injected with ghrelin, and such difference did not reach statistical significance in DAT-GHSR mice indicating that c-Fos is not an optimal marker for ghrelin-induced activation of DA neurons. We also found that c-Fos levels increase in the Acb of the DAT-GHSR mice in response to centrally-injected ghrelin, as seen in WT mice. Such observation may be another indication that VTA^{DA} neurons are directly activated by ghrelin since activation of the mesocorticolimbic pathway is known to induce a DA-dependent increment of c-Fos levels in striatal regions (Graybiel et al., 1990). In contrast to WT mice, however, ICV-injected ghrelin did not affect either regular chow intake, seeking or locomotor activity in DAT-GHSR mice. Thus, a direct action of ghrelin on midbrain DA neurons does not appear to mediate the acute stimulatory effects of centrally-injected ghrelin on these behaviors. Interestingly, local administration of ghrelin in the VTA increases both food intake and locomotor activity in mice, and blockade of DA receptors in the Acb does not affect chow intake induced by intra-VTA-injected ghrelin (Cornejo et al., 2018a; Skibicka et al., 2013). Furthermore, ICV-injected ghrelin increases c-Fos levels in non-DA cells of the VTA, including in a

subpopulation of GABA neurons (Cornejo et al., 2018a). In addition, centrally-injected ghrelin has been shown to indirectly activate the mesocorticolimbic pathway via other brain areas, such as the laterodorsal tegmental area or the lateral hypothalamic area, which also express GHSR (Jerlhag et al., 2007; Zigman et al., 2006). Thus, redundant neuronal circuits seem to be able to mediate the stimulatory effects of ghrelin on the mesocorticolimbic pathway. Future studies are still required to reveal the phenotype and localization of the non-DA neurons that mediate the stimulatory effects of centrally-injected ghrelin on food intake, food seeking and locomotor activity.

The behavioral implications of GHSR signaling become more evident under conditions of energy deficit, when plasma ghrelin levels and GHSR expression increase (Uchida et al., 2013). GHSR-deficient mice show a reduced compensatory hyperphagia in response to fasting and reduced anticipatory locomotor activity in response to scheduled meals, as compared to WT littermates (Blum et al., 2009; Fernandez et al., 2018). Calorie restricted GHSR-deficient mice also show impaired conditioned place preference to HF diet (Perello et al., 2010). Under energy deficit conditions, however, food intake depends not only on DA pathways but also on neuronal circuits controlling the homeostatic aspects of food intake (Liu et al., 2012; Perello and Dickson, 2015). In order to maximize our chances to unmask a role of GHSR in DA neurons, DAT-GHSR mice were studied using behavioral paradigms that involved: 1) *ad libitum* fed conditions, when neuronal circuits regulating the homeostatic aspects of food intake are presumably less active, and 2) a HF diet as a palatable stimulus since DA pathways are thought play a major role on the rewarding aspects of appetite (Bake et al., 2014).

Previous studies showed that male mice with free access to regular chow and daily-limited access to HF diet display increased locomotor activity preceding the scheduled palatable meal (Gallardo et al., 2012; Hsu et al., 2010). Interestingly, plasma ghrelin levels increase in anticipation to a palatable stimulus and positively correlate with food anticipatory activity, in *ad libitum* fed rats (Merkestein et al., 2012; Sirohi et al., 2017). Furthermore, centrally-injected ghrelin increases, whereas a GHSR antagonist decreases, the anticipatory activity towards chocolate (Merkestein et al., 2012). Thus, we decided to assess anticipatory activity to scheduled HF diet exposure in satiated DAT-GHSR mice. Since ghrelin treatment also increases the conditioned place preference to HF diet (Perello et al., 2010), we assessed anticipatory activity in a 2-chamber apparatus, in which HF diet was daily present in only one of the chambers. As predicted, GHSR-deficient mice displayed a reduced anticipatory activity to HF diet, as compared to WT mice. In contrast, DAT-GHSR mice showed an anticipatory activity to HF diet similar to that observed in WT mice. Notably, DAT-GHSR mice showed an impaired capability to associate the experience of HF intake with a

particular chamber, similar as observed in GHSR-deficient mice. Thus, GHSR signaling in DA neurons is sufficient to increase HF diet anticipatory activity whereas GHSR at other neuronal targets, different from DA neurons, appears to be important for some learning aspects of HF intake. A potential brain area mediating such GHSR-associated learning functions is the hippocampus, where the receptor is also present and can mediate orexigenic effects of ghrelin (Hsu et al., 2018). Overall, current results indicate that GHSR expression in DA neurons is sufficient to drive some, but not all, appetitive behaviors that take place in mice conditioned to receive a scheduled HF stimulus.

In order to test if GHSR in DA neurons regulates consummatory aspects of HF diet intake, satiated DAT-GHSR mice were studied under a simple experimental paradigm in which *ad libitum* fed mice display a robust intake of HF diet. In particular, mice were exposed to a HF pellet every morning for 2-h over 4 consecutive days, while maintaining free access to regular chow. Under these experimental conditions, binge-like HF intake is mainly controlled by the rewarding aspects of appetite and involves activation of the mesocorticolimbic pathway (Valdivia et al., 2015). Importantly, a full binge-like HF intake requires GHSR signaling since mice lacking GHSR eat less HF diet under this, or similar, experimental conditions (King et al., 2016; Valdivia et al., 2015). Here, we confirmed that WT mice daily and time-limited exposed to HF diet display binge-like eating events that gradually escalate over the accesses. In contrast, GHSR-deficient mice also binge on HF diet, but they show a smaller cumulative HF intake. Notably, DAT-GHSR mice showed a HF intake indistinguishable from WT mice indicating that GHSR expression exclusively in DA neurons is sufficient to orchestrate binge-like HF intake.

The selectivity afforded by the DAT-Cre mouse prevents to establish, with certainty, the DA pathway mediating GHSR effects on the control of appetitive and consummatory behaviors towards HF diet. However, a large body of evidence allows us to predict that VTA^{DA} neurons of the mesocorticolimbic pathway are a key candidate mediating GHSR actions on these aspects of HF intake. On one side, the mesocorticolimbic pathway has been systematically associated to rewarding aspects of food intake (Howick et al., 2017; Perello and Dickson, 2015). For instance, brain areas of the mesocorticolimbic pathway are activated in rats showing anticipatory activity to scheduled HF diet exposure (Mendoza et al., 2005). VTA^{DA} neurons excitability is enhanced by the repeated exposure to rewarding stimuli, and this enhanced DA signaling has been associated with increased motivation and intake escalation of these stimuli, as seen for HF diet (Steketee and Kalivas, 2011). Moreover, VTA^{DA} neurons are responsive to HF intake (Valdivia et al., 2014). On the other side, the mesocorticolimbic pathway is a well-established target of GHSR signaling

(Cornejo et al., 2018a; Perello and Dickson, 2015; Zigman et al., 2006). Indeed, intra-VTA-injected ghrelin increases DA release in the Acb as well as preference for and motivation to obtain HF diet (Abizaid et al., 2006; Jerlhag et al., 2007; King et al., 2011; St-Onge et al., 2016). A recent study reported that GHSR-deficient mice with virus-induced GHSR overexpression in the VTA showed increased response to cocaine and novelty stress (Skov et al., 2017). Unfortunately, appetitive and consummatory behaviors towards HF diet were not investigated in this mouse model, in which GHSR expression was induced in VTA neurons that were programed to express GHSR as well as in other VTA neurons that should not express GHSR. It is interesting to mention that some evidence show that SN^{DA} neurons of the nigrostriatal pathway can modulate some aspects of food intake, such as food anticipatory activity (Lartigue and McDougle, 2019). In addition, SN^{DA} neurons express GHSR and its firing rate as well as DA release in the dorsal striatum are increased in response to ghrelin (Andrews et al., 2009). Moreover, ghrelin treatment increases DA turnover in the dorsal striatum, and increases impulsivity (Anderberg et al., 2016). Thus, we cannot rule out that GHSR in SN^{DA} neurons mediate some of the food reward-related behaviors that are restored in DAT-GHSR mice, as compared to GHSR-deficient mice.

The present study shows that GHSR in DA neurons is sufficient to mediate specific appetitive and consummatory behaviors towards HF diet, in the absence of caloric needs. The observations that GHSR in DA neurons fully restores binge-like HF intake and the anticipatory activity to HF diet exposure but is insufficient to restore the acute orexigenic or locomotor effects of ghrelin treatment suggest that the mechanisms by which ghrelin controls the rewarding aspects of eating behaviors can be fully dissociated from those mechanisms controlling the homeostatic aspects of feeding. It is interesting to mention that we chose to use HF diet as a reinforcer based on our previous experience studying the role of ghrelin on the rewarding aspects of food intake. However, other studies have shown that GHSR signaling also enhances the rewarding value of other natural (e.g. sugar, sex) and artificial (e.g. artificial sweeteners, alcohol, cocaine) reinforcers (Perello and Dickson, 2015; Zallar et al., 2017). Thus, it can be hypothesized that GHSR expression in DA neurons may play a broader role increasing the sensitivity of the mesocorticolimbic system to rewarding stimuli in general. Future studies will be required to test this possibility and determine if GHSR has a stimulus-dependent or a general effect in the regulation of the mesocorticolimbic circuit.

The authors have nothing to disclose.

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Figure captions

Fig. 1. DAT-Cre mice show Cre expression exclusively in DA neurons. (A) representative photomicrographs of coronal sections of the ARC (upper row), VTA (middle row) and SN (bottom row) of DAT-tdTomato mice immunostained against TH. Left column shows tdTomato+ neurons (red), middle columns shows TH+ neurons (green) and right column shows the merge of red and green signals. In each image, a schematic delimitation of the ARC, VTA and SN is overlaid according to the mouse brain atlas (Paxinos and Franklin, 2001). Insets show, in high magnification, the areas delimited by a rectangle in low magnification images. Arrows point to dual tdTomato+/TH+ cells. Scale bars: 10 µm and 100 µm for low and high magnification images, respectively. (B) and (C), quantitative analysis of GHSR expression in the ARC (B) and the VTA/SN (C) of the different experimental groups. Bar graphs represent GHSR relative expression quantified by qRT-PCR and normalized using the L19 ribosomic protein gene as the housekeeping. One-way ANOVA (ARC: $F(2,9)=26.24$, $p=0.0002$; VTA/SN: $F(2,9)=28.85$, $p=0.0001$) followed by Tukey's multiple comparisons test (*, $p<0.05$ vs WT and DAT-GHSR).

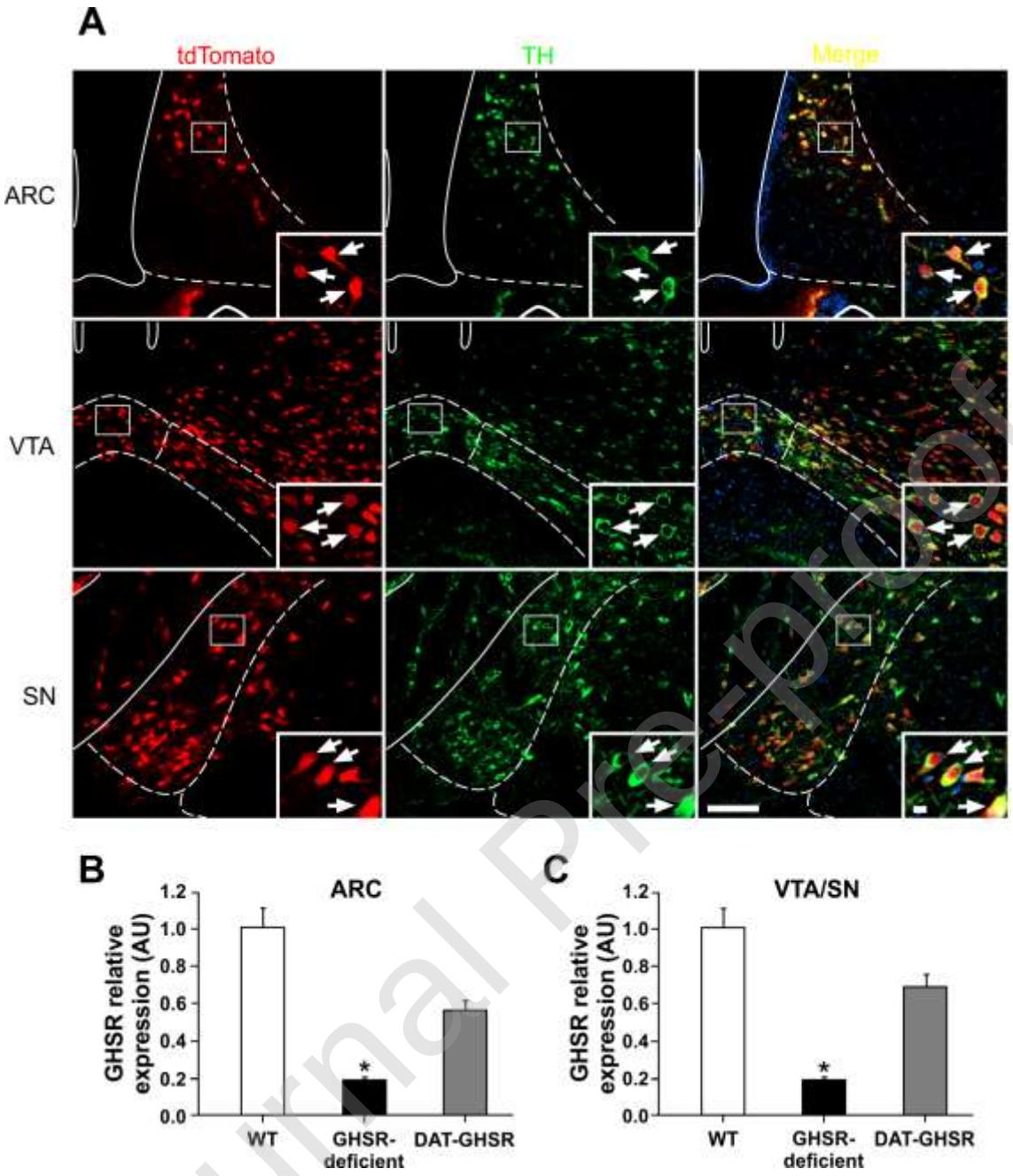

Fig. 2. DAT-GHSR mice do not increase food intake nor locomotor activity in response to SC- or ICV-injected ghrelin. (A) shows the quantitative analysis of 2-h food intake of mice SC-injected with either vehicle or ghrelin. Two-way ANOVA ($F(2,48)_{genotype}=5.073$, $p=0.0100$; $F(2,48)_{interaction}=6.736$, $p=0.0026$) followed by Bonferroni's multiple comparisons test (*, $p<0.05$ vs vehicle same genotype). (B) shows the quantitative analysis of 2-h food intake of mice ICV-injected with either vehicle or ghrelin. Two-way ANOVA ($F(1,56)_{treatment}=11.64$, $p=0.0012$; $F(2,56)_{genotype}=5.635$, $p=0.0059$ and $F(2,56)_{interaction}=16.45$, $p<0.0001$) followed by Bonferroni's multiple comparisons test (*, $p<0.05$ vs vehicle same genotype). (C) shows the quantitative analysis of the time spent digging

by mice ICV-injected with either vehicle or ghrelin. Two-way ANOVA ($F(1,25)_{treatment}=4.757$, $p=0.0388$; $F(2,25)_{genotype}=3.688$, $p=0.0395$ and $F(2,45)_{interaction}=10.59$, $p=0.0005$) followed by Bonferroni's multiple comparisons test (*, $p<0.05$ vs vehicle same genotype). (D) shows the quantitative analysis of the locomotor activity of mice ICV-injected with either vehicle or ghrelin. Two-way ANOVA followed by Bonferroni's multiple comparisons test (*, $p<0.05$ vs vehicle same genotype).

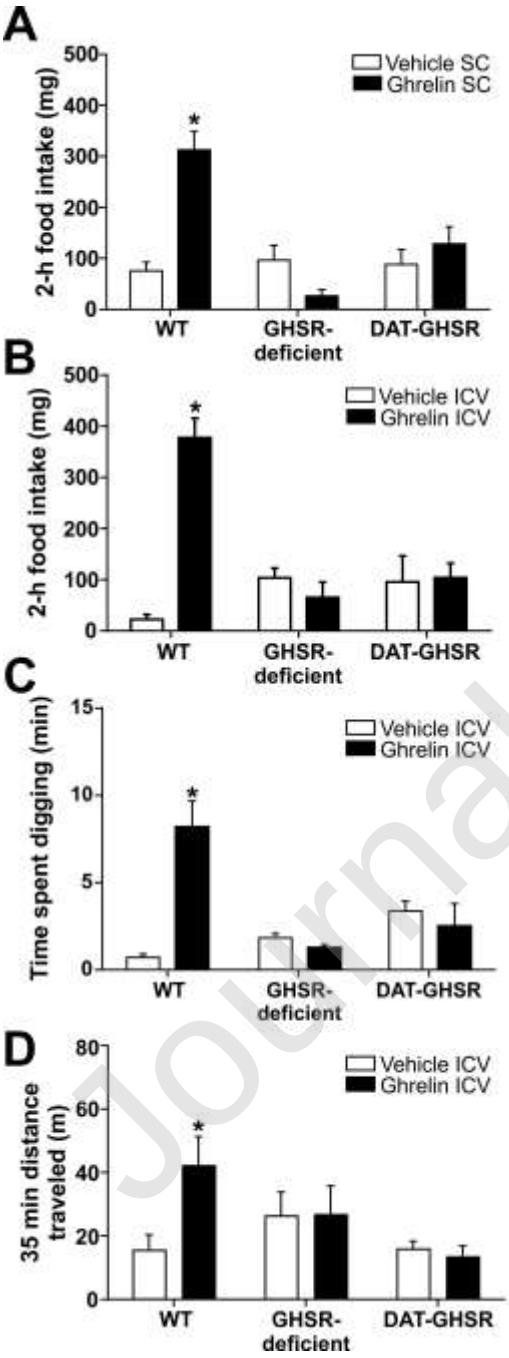

Fig. 3. DAT-GHSR mice show activation of DA brain nuclei in response to ICV-injected ghrelin. (A) shows representative photomicrographs of coronal brain sections containing the ARC, VTA, SN and Acb (from top to bottom) of WT (left column), GHSR-deficient (middle column) and DAT-GHSR (right column) mice ICV-injected with ghrelin subjected to a double immunohistochemistry against c-Fos and TH. In each image, a schematic delimitation of the ARC, VTA, SN and Acb is overlaid according to the mouse brain atlas (Paxinos and Franklin, 2001). Insets show, in high magnification, the areas delimited by a rectangle in low magnification images. Arrows point to c-Fos/TH+ cells and arrowheads, to c-Fos+ nuclei. (B)-(E) show the quantitative analysis of the number of c-Fos+ cells in the ARC (B), VTA (C), SN (D) and Acb (E) of mice ICV-injected with either vehicle or ghrelin. For the ARC: two-way ANOVA, $F(2,49)_{genotype}=6.032$, $p=0.0046$; $F(1,49)_{treatment}=19.39$, $p<0.0001$ and $F(2,49)_{interaction}=4.466$, $p=0.0165$; Dunnett's T3 multiple comparisons test: *, $p<0.05$ vs vehicle same genotype. For the VTA: two-way ANOVA $F(1,49)_{treatment}=15.66$, $p=0.0002$; Dunnett's T3 multiple comparisons test: *, $p<0.05$ vs vehicle same genotype. For the SN: two-way ANOVA $F(1,49)_{treatment}=12.22$, $p=0.0010$; $F(2,49)_{interaction}=3.420$, $p=0.0407$; Dunnett's T3 multiple comparisons test: *, $p<0.05$ vs vehicle same genotype. For the Acb: two-way ANOVA, $F(1,49)_{treatment}=12.57$, $p=0.0009$; Dunnett's T3 multiple comparisons test: *, $p<0.05$ vs vehicle same genotype.

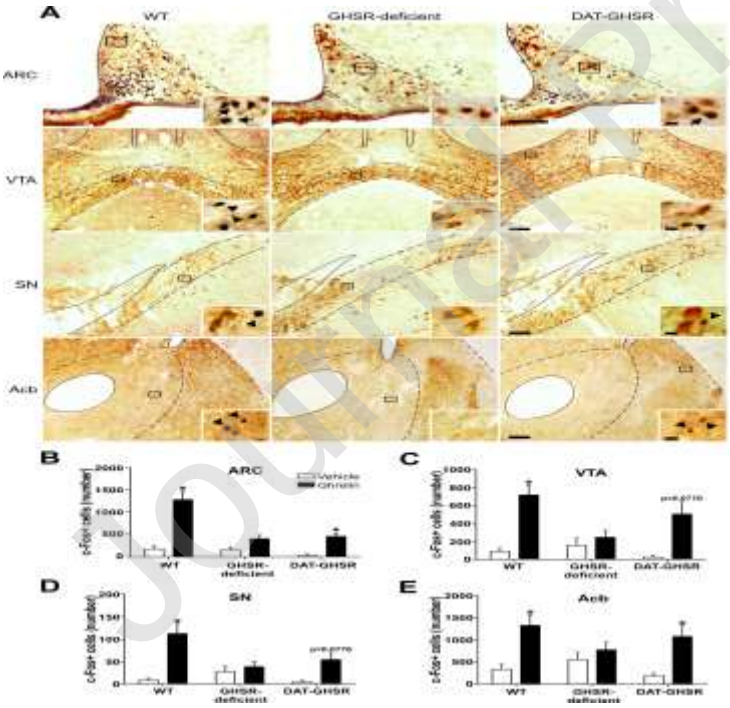

Fig. 4. DAT-GHSR mice display HF diet seeking and intake similar as WT mice. (A) shows a schematic representation of the experimental design of the HF diet seeking protocol. (B) and (C) show the quantitative analysis of the total distance traveled (B) and the time spent in the empty

and the HF diet-paired side (C) of WT, GHSR-deficient and DAT-GHSR mice subjected to the HF diet seeking protocol. For the total distance traveled: one-way ANOVA ($F(2,27)=4.061$, $p=0.0287$) followed by Holm-Sidak's multiple comparisons test (*, $p<0.05$ vs WT and DAT-GHSR); for the time spent in each chamber side: two-way ANOVA ($F(2,54)_{interaction}=7.598$, $p=0.0012$) followed by Sidak's multiple comparisons test (*, $p<0.05$ vs empty chamber). (D) shows a schematic representation of the experimental design of the HF binge eating protocol. (E) and (F) show the quantitative analysis of the 2-h HF diet intake (E) and the cumulative 4-days HF diet intake (F) of WT, GHSR-deficient and DAT-GHSR mice exposed to the HF diet binge eating protocol. For the 2-h HF diet intake: two-way RM ANOVA $F(3,156)_{time}=68.13$, $p<0.0001$; $F(2,52)_{genotype}=4.407$, $p=0.0171$ followed by Bonferroni's multiple comparisons test (a, $p<0.05$ vs day 1 same genotype; b, $p<0.05$ vs WT same day). For the cumulative 4-days HF diet intake: one-way ANOVA ($F(2,54)=4.062$, $p=0.0227$) followed by Holm-Sidak´s multiple comparisons test (*, $p<0.05$ vs GHSR-deficient).